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A 24-bp Indel (Insertion-Deletion) Polymorphism in Promoter Prolactin Gene of Papua Local Chickens

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Abstract. Prolactin in chicken (cPRL) is a polypeptide hormone that plays a crucial role in incubation and brooding behavior. This hormone is coded by a gene located at chromosome 2. The objective of this study was to investigate 24-bp indel polymorphism in promoter prolactin gene of Papua local chickens. A number of 60 DNA samples came from 60 Papua local chickens (25 males and 35 females) were used in this study. PCR (*Polymerase Chain Reaction*) method with a pair of specific primer was applied and a specific DNA fragment with 130 and/or 154 bp contained 24-bp indel was successfully amplified. Results of electrophoresis run on 1.5 % agarose gel showed three genotypes: II, ID and DD with the frequency of 6,7%, 48.3% and 45% respectively. The frequency of I allele and D allele was 0.69 and 0.31, respectively. Results indicated that the I allele frequency was medium in Papua local chickens population. Results therefore suggest that incubation behavior in hen population of Papua local chickens is easy to eliminate by increasing the I allele frequency in the population.

Key words: 24-bp Indel, Papua local chicken, polymorphism, promoter prolactin gene.

Abstrak. Prolaktin pada ayam (cPRL) merupakan hormon polipeptida yang memainkan peranan penting terhadap tingkah laku mengeram. Hormon ini disandi oleh sebuah gen yang terletak pada kromosom 2. Tujuan penelitian ini adalah untuk mendeteksi polimorfime 24-bp Indel pada daerah promoter gen prolactin (cPRL) pada ayam lokal Papua. Enam puluh sampel DNA genom yang berasal dari 60 ekor ayam lokal Papua (25 jantan dan 35 betina) digunakan dalam penelitian ini. Dengan menggunakan metode PCR (*Polymerase Chain Reaction*) dan sepasang primer spesifik telah berhasil diamplifikasi fragmen DNA spesifik (ukuran 130 atau 154 bp) yang mengandung 24-bp indel tersebut. Hasil running elektroforesis pada gel agarose 1,5% ditemukan 3 genotip, yaitu II, ID dan DD, dengan frekuensi berturut-turut sebesar 6,7%, 48,3%, dan 45%, dan frekuensi alel I dan D sebesar 0,31 dan 0,69. Hasil temuan ini menunjukkan frekuensi alel I tergolong sedang dalam populasi ayam lokal Papua. Keadaan ini menunjukkan bahwa perilaku mengeram pada populasi induk ayam lokal Papua mudah dieliminir dengan cara meningkatkan frekuensi alel I dalam populasinya.

Kata kunci: 24-bp Indel, ayam lokal Papua, polimorfisme, promoter gen prolaktin

Introduction

Papua local chickens are non-commercial breed found in Papua land, and they become one of Indonesia germplasms. These chickens have been considered the primary commodity due to their contributions in welfare improvement of the local society in the region. Besides their high variability in body weight (Lumatauw et al., 1995; Haryani, 1999; Mu'in, 1999) and carcass percentage (Rahayu, 1990), the Papua local chickens also show high variability in the number and egg weight produced in every clutch (Lebang, 2002). This indicates that the production and reproduction performance of Papua local chickens are easy to improve through genetic selection.

Today, with the advance technology in molecular biology, genetic improvement of an animal population can be done through molecular approach. Polymorphism detection on the loci of protein-coded gene associated with economically traits of animals is made possible. If a polymorphic loci associated with economically traits of animal is found, it therefore can be used as molecular marker in selection program to improve specific trait.

Prolactin is a peptide hormone secreted by the anterior pituitary gland and it has a wide role in the activities and biological function in all vertebrates. In birds, prolactin hormone (cPRL) has a crucial role in egg production and brooding behaviour due to the increase of prolactin secretion (Shimada et al., 1991; Talbot and Sharp, 1994) that results in regression of the ovary (Sharp et al., 1984) therefore, stop the egg production (Shimada et al., 1991).

In birds, cPRL is coded by a gene located on chromosome 2 (Miao et al., 1999; Au and Leung, 2000), and become a candidate gene for brooding trait (Shimada et al., 1991; Dunn et al., 1998) and egg production (Cui et al*.,* 2006). Chicken prolactin gene is 9.536 bp in size, consisted of 3 parts, promoter-1, promoter-2 and promoter-3 with 330 bp, 287 bp, and 314 bp, respectively (Kansaku, 2000). Five exons of cPRL are exon-1 (81 bp), exon-2 (182 bp), exon-4 (180 bp) (Ohkubo et al., 1998), exon-3 (59 bp) (Miao et al., 1999), and exon-5 (418 bp) (Cui et al., 2004). The intron parts are intron-1 (714 bp), intron-2 (406 bp), intron-4 (744 bp) (Dhara and Soller, 1999). The size of intron-3 remains unknown.

Promoter of cPRL is located at the start point and become crucial due to its early activation function for transcription of cPRL gene expression (Lewin, 1997). Mutation that occurs on the promoter region causes the cPRL gene fails to express its product and unable to express the brooding behaviour Therefore, the egg production will increase.

It has found several mutations of cPRL promoter region, and one is 24-bp (Cui et al., 2006; Liang et al., 2006; Rashidi et al., 2012). Indel on -358 site where Insertion (I) or Deletion (D) is presence. There are two alleles of this 24-bp locus: I and D alleles. In non-commercial breed of chickens, the I allele frequency vary from low to medium (Cui et al., 2006; Begli et al., 2010; Rashidi et al., 2012), while in the commercial layer, the I allele is commonly found. In their study, Cui et al (2006) did not find any other allele except the I allele in White Leghorn. In chicken, 24-bp Indel polymorphic is known significantly associated with egg production (Cui et al., 2006; Begli et al., 2010; Rashidi et al., 2012). These findings give an opportunity for breeders to form and develop a future layer type of the Papua local chickens.

The objective of this study is to get information regarding the allele frequency and genotype of 24-bp Indel in promoter cPRL gene of Papua local chickens. This information therefore, will be useful in the formation and development of layer type of the Papua local chicken in the future.

Materials and Method

Blood Samples

Sixty DNA samples isolated from blood samples of sixty Papua local chickens were used in this study. The experimental chickens that consisted of 25 males and 35 females were randomly collected from several farmers in Manokwari Regency of West Papua Province. About 1 ml of blood sample was collected from each bird via brachialis venous using a 1 ml disposable syringe and stored in a 3 ml vacuum tube contained K_3EDTA. The blood samples then were carried to the Biochemistry Laboratory of Biotechnology Study Centre, Gadjah Mada University for DNA isolation and DNA analysis.

DNA Isolation

DNA samples isolation of experimental birds was carried out by applying *phenol-chloroform* extraction method (Sambrook et al., 1989). The procedure was as follows: First, as much as 1 ml blood sample was transferred from *vacutainer* tube to a 15 ml sterile *conical* tube and added up to 15 ml with PBS. PBS is a solution contained 8g NaCl, 0.2g KCl, 1.44g $NaHPO_4.2H_2O$, 0.2g KH_2PO_4 that was added up with dH_2O to 1 liter. The solution was centrifuged at 3000 rpm for 15 min. The supernatant was discarded while the pellet was re-suspended with 1-2 ml PBS. This step was done three times. The last pellet was re-

suspended with TE solution then added it up to 5 ml.

K Proteinase solution (1 mg in NTE pH 7.4) was prepared and let in 37°C *waterbath* for 15 min. The NTE, pH 7.4 is a buffer contained 20 mL 5 M NaCl, 20 mL 1 M Tris-KCl (pH 7.4), 2 mL 0.5 M EDTA (pH 7.5) and 958 mL dH_2O. As much as 50 μL Proteinase K 10 mg/mL was added into 100 μg/mL. 250 μL of SDS 10 % was added and the last concentrations become 0.5%, incubated in the *water bath* for an overnight in 37°C. 1:1 Phenol (±5 mL) was added, shaked for 20 min, and centrifuged at 3000 rpm for 20 min. The supernatant was transferred into a new and sterile 15 mL *conical* tube and as much as 1:1 (± 5 mL) of CIAA (24:1) was added and *vortex*, centrifuged (3000 rpm; 20 min). The supernatant was transferred again into another new and sterile 15 mL conical tube and Na Acetate 3 M pH 4.8 with 1/10 was added. About 10 mL cool absolute Ethanol with the ratio of 2:1 was added, slowly inverted until thread like DNA appeared.

The solution contained DNA was centrifuged (3000 rpm; 15 min), and the supernatant was discarded. Pellet located on the bottom was rinsed with 70% ethanol and let dry. TE was added according to the size of pellet and let overnight until the DNA was completely dissolved in TE.

The DNA samples obtained from the result of the isolation was measured for its concentration and purity with the following procedures: 2 μL of DNA solution was mixed with 98 μL distilled water in a 1,5 mL micro tube and examined under spectrophotometer at 260 nm and 280 nm wavelength. The double strands DNA concentration was equal to 50 μg/mL when OD_{260} value of the measured DNA was equal to one. The DNA purity was measured by dividing the OD_{260} with OD_{280}. The DNA molecule is said to be pure when the ratio between the two values is around 1.8 to 2.0 (Muladno, 2002).

DNA Amplification and Genotyping

Specific DNA fragment amplification that contained the studied loci was conducted with the *Polymerase Chain Reaction* (PCR) technic. The primer used to amplify the specific DNA fragment was *forward*: 5'-TTT-AAT-ATT-GGT-GGG-TGA-AGA-GAC-A-3', and *reverse*: 5'-ATG-CCA-CTG-ATC-CTC-GAA-AAC-TC-3' (Cui *et al*., 2006).

The DNA amplification process was initiated by adding 19 μL dH_2O, 2 μL DNA solution and 2 μL each for the *forward* and *reverse* primers into *Pure Tag™ Ready-To-Go™ PCR Beads* tube (Amersham Biosciences).

PCR condition of amplification for 130 and/or 154 bp specific fragment DNA contained 24-bp indel on –358 site of cPRL promoter-3 gene was done according to Cui et al. (2006) as follow: Initial denaturation at 94°C for 5 min, continued with amplification for 35 cycles (denaturized at 94°C for 30 s, annealing at 54°C for 30 s, 72°C extention for 30 s), and final extension on 72°C for 5 min. PCR optimization was conducted to find the ideal annealing temperature.

PCR product (size: 130 bp and/or 154 bp) got from the amplification result was directly electrophoresed on 1,5% agarose gel contained Gold Nucleic Acid Stain in TBE buffer. The procedure was: 5 μL of amplicon was mixed with 2 μL loading buffer, and then was put in gel well. *Running* gel was done at 100 volts for 30 min together with DNA marker (size 100-3.000 bp). Picture of the resulted electrophoresis was taken with a digital camera.

Genotype identification of 24-bp Indel/cPRLp was as follow: II genotype (*Insertion-Insertion*) characterized by one DNA fragment size 154 bp; ID genotype (*Insertion-Deletion*) characterized by two DNA fragments size 154 bp and 130 bp; and DD genotype (*Deletion-Deletion*) characterized by one DNA fragment size 130 bp (Cui et al., 2006).

Data Analysis

Data of genotype and alleles of the studied locus (24-bp Indel/cPRLp) on Papua local chickens were estimated for their frequencies by using Nei and Kumar procedure (2000).

$X_{ii} = n_{ii}/n$ $X_i = (2n_{ii} + \Sigma n_{ij}) /(2n)$

where:

X_{ii} = genotype frequency of the ii-th
X_i = allele frequency of the i-th
n_{ii} = number of ii genotypies
n_{ij} = number of ij genotypies
n = number of samples

Locus was polymorphic when the common allele frequency found in the population did not exceed 99% (Nei and Kumar, 2000).

Results and Discussion

Prolactin gene in chicken (cPRL) specifically on the promoter region is a candidate gene for brooding behaviour (Shimada et al., 1991; Dunn et al., 1998), and egg production (Cui et al., 2006). cPRL promoter gene is an important part that responsible in the expression or the function of cPRL. The position of promoter in cPRL gene is located at the *starting point* (Lewin, 1997) and has its role in activating the early transcription for the gene expression. If a mutation occurs in this promoter region, the cPRL gene will not function and fail to express its product, thus, the brooding behaviour will not appear.

Specific DNA fragment (size 130 bp and/or 154 bp) amplification located at the promoter region, contained 24-bp Indel on -358 site that flanked by a pair of specific primers (Cui et al., 2006) has demonstrated by using PCR (*Polymerase Chain Reaction*) in 60 DNA samples of Papua local chickens. The result of amplification showed three genotypes II, ID and DD (Figure 1). The chickens with II genotype were 4 birds, chickens with ID genotype were 29 birds and chickens with with DD genotype were 27 birds.

The three genotypes found in this study were as the result of the presence of mutation on the promoter region. The polymorphism of 24-bp Indel on -358 site was due to the presence of insertion (I) and or deletion (D) as many as 24 bp (Cui et al., 2006; Liang et al., 2006; Rashidi et al., 2012). Sequence of DNA fragment size 154 bp (I allele) and size 130 bp (D allele), presented in Figure 2 and 3, respectively.

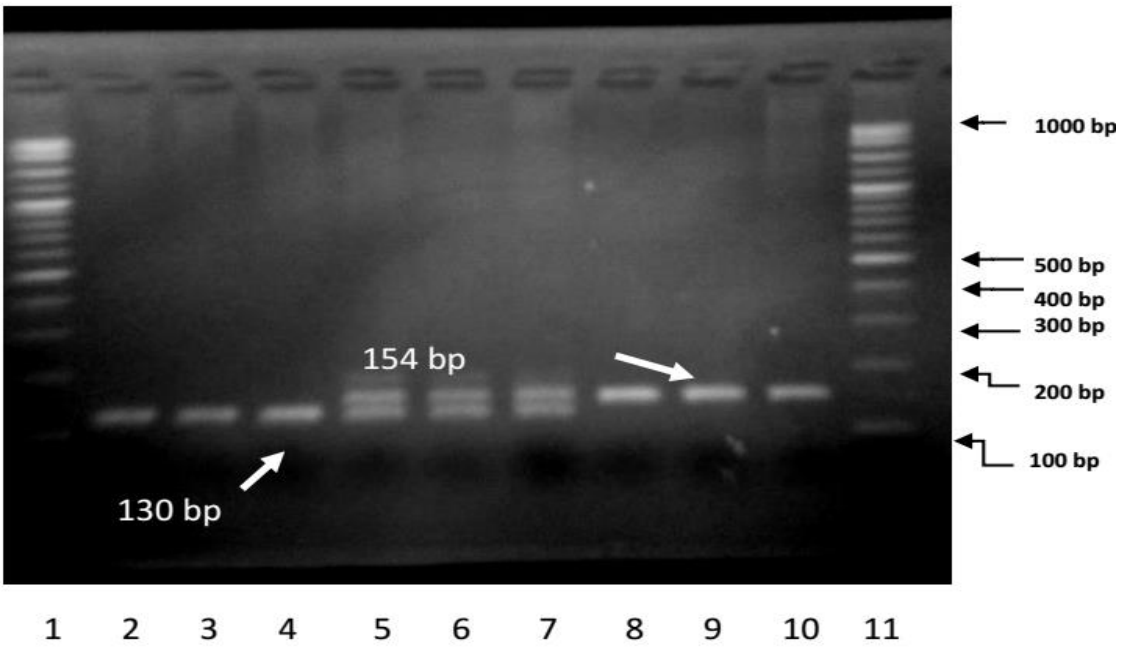

Figure 1. Genotypes of the 24-bp indel at np 358 by PCR with agarose gel electrophoresis in Papua local chickens. Lanes 1 and 11: DNA marke (100 – 3000 bp); lanes 2 – 4: DD (deletion-deletion, size: 130 bp); lanes 5 – 7: ID (insertion-deletion, size: 130 bp and 154 bp); and lanes 8 – 10: II (insertion-insertion, size: 154 bp).

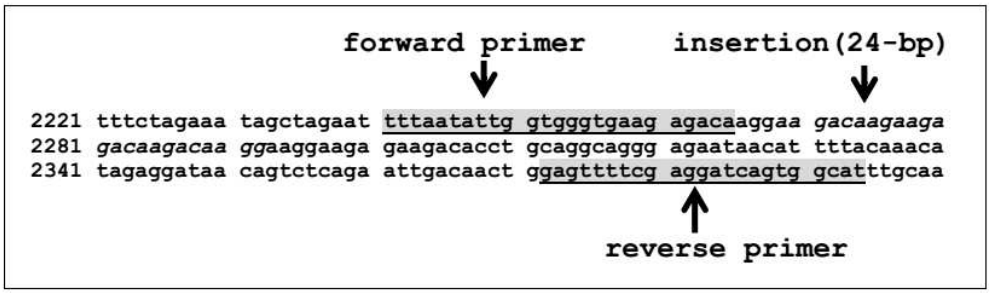

Figure 2. Sequence of DNA fragment in gallus gallus prolactin gen (promoter region), 154 bp (insertion allele). Source: GenBank: AB011438.2

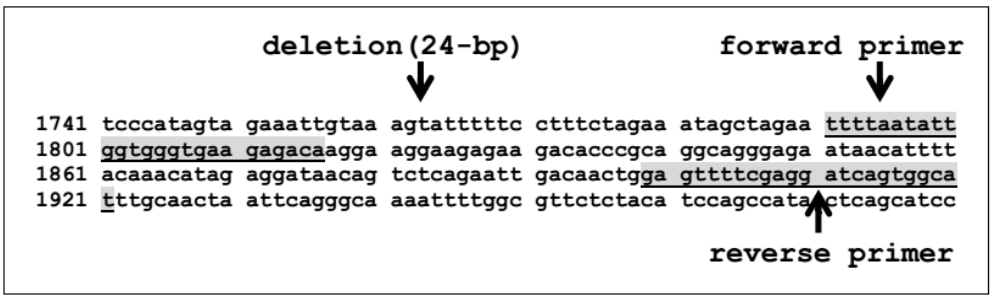

Figure 3. Sequence of DNA fragment in gallus gallus prolactin gen (promoter region), 130 bp (deletion allele). Source: GenBank: AF288765.2

Results for allele frequency and genotype calculations of 24-bp Indel/ cPRLp on Papua local chickens that were estimated by using the Nei and Kumar's procedure (2000) showed that II, ID and DD genotype frequencies were 6,7%, 48.3%, and 45%, respectively while the I and D alleles were 0.31 and 0.69. The I frequency of 0.31 found in the Papua local chickens was considered medium. This is in accordance with Cui et al. (2006), Begli et al. (2010), and Rashidi et al. (2012) that the I frequency on non-commercial breed (local chickens) varied from low to medium, while on commercial breed (layer type), the I allele was common (common allele). In 2006, Cui et al did not find any other allele except the I allele on *White Leghorn*.

Based on the above research information, it was known that the presence of I allele (Insertion) in chickens gives positive effects on traits related to egg production. On the other hand, the presence of the D allele (Deletion) in chickens tends to give negative effects on egg production. Indel (Insertion-Deletion) is a term in molecular biology that has different definitions on several aspects. In the study of evolution, indels are used to bear a meaning for *an insertion* event (insert or interpolation) and or *deletion* (delete) (Kondrashov and Rogozin, 2004; Ogurtsov et al., 2004). An indel can change the DNA sequence and form a *frame shift,* therefore, change in amino acids sequence produced, thus the produced protein become abnormal even no protein is produced.

Indels can be used as a genetic marker in a population especially in phylogenetic study (Erixon and Oxelman, 2008). In the study of mutation of sex and somatic cells, indel is described as a specific mutation that adds or deletes part of nucleotide sequence. An indel is categorized small when the mutation produces additional or deletion of 1 to 50 nucleotides (Gonzalez et al., 2007). Indels usually do not occur on the *coding region* but they commonly occur on the *non-coding regions*. Indel is different from point mutation where indel insert or delete part of nucleotide sequence while point mutation is a form of nucleotide

substitution that change one with other nucleotide.

In chickens, an 24-bp Indel (*Insertion-Deletion*) polymorphism is known significantly associated with egg production (Cui et al., 2006; Begli et al., 2010; Rashidi *et al*., 2012). The presence of medium frequency of I allele found in Papua local chickens in this study indicated that the formation of Papua local chicken population that are high in egg production become easy to realized through a controlled mating application within the Papua local chickens with II and ID genotypes without chickens with DD genotypes involved. Therefore, the brooding trait in Papua local chickens will slowly disappeared and in turn, a high egg production of Papua local chicken will be formed.

Several researches of 24-bp indel on the promoter region gene in cPRL of several breeds or genetic groups of chickens and its effects on quantitative traits had been demonstrated. Cui et al. (2006) carried out a research to study polymorphism on the promoter region gene in cPRL of several breeds of chickens and studied their effects on egg production. Genotyping of polymorphic 24-bp indel (insertion-deletion) loci on -358 site of cPRL gene was applied on 177 chickens that consisted of White Lenghorn, Yangshan, Taihe Silkies, White Rock, and Nongdahe. Results showed that the I allele (*Insertion*) and the D (*Deletion*) frequencies were 1 and 0; 0.05 and 0.95; 0.20 and 0.80; 0.22 and 0.78; 0.17 and 0.83, respectively. In the further analysis found that a polymorphic locus of 24-bp indel was significantly associated with the egg production where the presence of I allele gave positive effect on egg production. Jiang et al. (2005) informed their study that chickens with homozygote insertion (II genotype) of 24-bp on promoter region of cPRL could reduce the cPRL expression so that the chickens showed no brooding trait.

It was concluded that the cPRL promoter could be used as genetic marker for brooding trait in chickens. Rashidi et al. (2012) studied the native chickens of Iranian and found that the allele frequencies of polymorphic 24-bp loci indel of cPRL were 0.59 for the I and 0.41 for the D allele. The frequencies of II, ID and DD genotypes were 0.39; 0.40; and 0.21, respectively. As comparison, result on polymorphic 24-bp Indel on this promoter region in Quail showed that the I and D alleles frequency were almost balance, 0.52 and 0.48 (Lotfi et al., 2013).

Conclusions

Polymorphism of 24-bp indel on the promoter region of prolactin gene was detected in Papua local chickens. The I allele frequency was 0.31 and categorized medium. This indicated that the brooding trait on hen population of Papua local chickens could be eliminated by increasing the I allele in the population through a controlled mating.

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